Cell Metabolism
Short Article

PP2A Regulatory Subunit PP2A-B′ Counteracts S6K Phosphorylation

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DOI 10.1016/j.cmet.2010.03.015

SUMMARY

The insulin/TOR signaling pathway plays a crucial role in animal homeostasis, sensing nutrient status to regulate organismal growth and metabolism. We identify here the *Drosophila* B′ regulatory subunit of PP2A (PP2A-B′) as a novel, conserved component of the insulin pathway that specifically targets the PP2A holoenzyme to dephosphorylate S6K. *PP2A-B′* knockout flies have elevated S6K phosphorylation and exhibit phenotypes typical of elevated insulin signaling such as reduced total body triglycerides and reduced longevity. We show that PP2A-B′ interacts with S6K both physically and genetically. The human homolog of PP2A-B′, PPP2R5C, also counteracts S6K1 phosphorylation, indicating a conserved mechanism in mammals. Since S6K affects development of cancer and metabolic disease, our data identify PPP2R5C as a novel factor of potential medical relevance.

INTRODUCTION

Insulin signaling is an evolutionarily conserved pathway crucial for the proper regulation of growth, metabolism, and longevity in animals (Goberdhan and Wilson, 2003; Taguchi and White, 2008). Insulin signaling is highly conserved from mammals to flies, both molecularly and in terms of the physiological processes that it regulates (Grewal, 2009). Insulin signaling integrates inputs from growth factors and nutrients to regulate cellular metabolic pathways so that the proper organismal balance of nutrient/energy expenditure versus storage is achieved. Dysregulation of insulin signaling in humans contributes to disease, as hyperactivation of the pathway is observed in almost all cancers (Hafen, 2004), and reduced activation is associated with diabetes and obesity (Goberdhan and Wilson, 2003). (Although there are multiple connections between insulin and TOR signaling, the significance of these connections is currently an issue of debate. We use here the term "insulin signaling" in a broad sense, denoting insulin signaling and TOR signaling combined.)

In order to maintain organismal energy homeostasis, insulin signaling needs to respond to changing nutrient conditions, both rapidly increasing and decreasing its state of activation as necessary. The insulin pathway contains a large number of kinases that activate each other through a relay of phosphorylation events upon binding of insulin to its receptor. This gives a molecular explanation for activation of the pathway; however, the fact that the pathway can also be inactivated suggests phosphatases exist to counteract the various kinases.

S6K is an important effector of the insulin/TOR pathway inducing ribosome biogenesis, enhancing translation of mRNAs containing structured 5′UTRs and inhibiting apoptosis (Hay and Sonenberg, 2004). As a consequence, S6K promotes cell growth and proliferation, contributing to the development of cancer (Jastrzebski et al., 2007), and plays an important role in the regulation of organismal metabolism (Um et al., 2004). Thus, the precise regulation of S6K activity is critical for normal animal growth and homeostasis. Activation of S6K has been extensively studied and occurs via phosphorylation by TOR and PDK1, thus integrating nutrient availability and growth factor signaling (Jastrzebski et al., 2007). Dephosphorylation of S6K is less well understood. Inhibition of TOR causes S6K to become dephosphorylated within minutes, despite S6K protein levels remaining unchanged (Bielinski and Mumby, 2007), suggesting that a phosphatase dephosphorylates S6K.

We identify here PP2A-B′ (CG7913), encoding a regulatory B′ subunit of PP2A, as a specific negative regulator of S6K phosphorylation. PP2A-B′ knockout flies have elevated levels of S6K phosphorylation. They display significant metabolic aberrations, which are rescued by reducing S6K gene dosage. We show that PP2A-B′ mutants lose robustness to nutritional variation, highlighting an important role of PP2A-B′ in the maintenance of organismal homeostasis. Knockdown of the human PP2A-B′ homolog PPP2R5C in cells also affects S6K phosphorylation, implying functional conservation of this regulatory subunit in humans. Although the catalytic subunit of PP2A has been implicated in the dephosphorylation of S6K in cell culture (Ballou et al., 1988; Bielinski and Mumby, 2007), its function is highly pleiotropic, accounting for a large fraction of phosphatase activity in eukaryotic cells (Millward et al., 1999). The discovery of

a regulatory subunit targeting PP2A activity specifically to S6K opens the possibility of using it for diagnostic or therapeutic purposes.

RESULTS

PP2A-B′ Knockout Flies Are Lean and Short Lived

To study the function of PP2A-B′, we created *PP2A-B′* knockout flies by replacing the first exon common to all splice variants with the miniwhite gene by homologous recombination (Figure 1A) (Gong and Golic, 2003). This leads to premature disruption of the coding sequence and a shift in the reading frame of remaining exons, generating a null mutation. To clean the genetic background of knockout flies, females heterozygous for the knockout mutation were backcrossed to w^{1118} flies, an inbred but not isogenic laboratory stock. After four generations of backcrossing, stocks were established from single males, yielding two independent knockout lines, KO1 and KO2.

KO1 flies are viable to adulthood (Figure 1B), normal in developmental rate (Figure 1C), and almost normal in size (Figure 1D, see Figures S1A and S1B online). To test whether PP2A-B′ affects fly metabolism, we measured total body triglycerides, normalized to total body protein, of KO1 and w^{1118} control flies grown under controlled nutrient and density conditions. *PP2A-B′* mutants are strikingly lean, containing 28% of the triglyceride levels of w^{1118} controls (Figure 1E). This leanness is specific for loss of *PP2A-B′*, as it is significantly rescued by expression of *PP2A-B′* from a transgene (Figure 1E). *PP2A-B′* mutants also contain roughly half as much total body glycogen as control flies (Figure S1C). We also asked whether PP2A-B′ affects longevity and found that KO1 flies have significantly reduced life span compared to controls (Figure 1F).

We studied the effect of *PP2A-B′* overexpression by generating *UAS-PP2A-B′* transgenic lines and crossing them to GAL4 drivers. Overexpression of *PP2A-B′* with *en-GAL4* caused lethality with most UAS lines (data not shown). One weak UAS line, however, gave viable adults with wing posterior compartments—where *en-GAL4* is expressed—that were normally patterned but significantly reduced in size by 12% (Figure 1G). Overexpression of *PP2A-B′* in the head and eye using eyeless-Gal4 also caused a reduction in tissue size, ranging in strength from mild to strong (Figures 1H and 1H′). In summary, *PP2A-B′* overexpression causes a reduction in tissue size or lethality.

PP2A-B′ Represses S6K Phosphorylation

The phenotypes described above suggested PP2A-B′ might play a repressive role in insulin signaling. Flies with elevated insulin signaling in the whole body by mutation of *PTEN* have reduced lipid levels (Oldham et al., 2002), similar to *PP2A-B′* mutants. Insulin signaling also regulates longevity (Taguchi and White, 2008), and overactivation of S6K leads to reduced life span (Kapahi et al., 2004), similar to *PP2A-B′* mutants. Finally, inhibition of insulin signaling leads to reduced tissue growth (Goberdhan and Wilson, 2003), analogous to what we observe with *PP2A-B′* overexpression. Therefore, we asked whether PP2A-B′ affects insulin signaling by assaying phosphorylation of S6K, a direct target of TOR Complex 1 (TOR-C1) (Hay and Sonenberg, 2004). Immunoblotting of protein extracts from KO1 and control w^{1118} animals revealed that KO1 flies have significantly elevated levels of phosphorylated S6K (Figure 2A, lanes 1 and 2). This effect was reversed by ubiquitous expression of *PP2A-B′* from a transgene in the KO1 mutant background (Figure 2A, lane 3). Total levels of dERK are shown as loading control, since work in our laboratory with other genes of the insulin pathway has shown that tubulin levels can vary when insulin signaling is modulated (data not shown).

We asked at what level of the insulin pathway PP2A-B′ might be functioning by detecting the state of activation of other components of the pathway. We assayed phosphorylation of Akt on the site for which antibodies are available (S505) and found that it was unaffected in KO1 animals compared to controls (Figure 2B). We also assayed activity of the transcription factor FOXO, which is regulated via phosphorylation by Akt and is a sensitive readout for Akt activation (Puig et al., 2003). Quantitative RT-PCR on RNA extracted from KO1 and control w^{1118} animals revealed no difference in mRNA levels of a well-characterized FOXO target, *4E-BP* (Figure 2C) (Teleman et al., 2005). These data indicate that activation of the pathway at the level of Akt is not increased in *PP2A-B′* mutants. If anything, we observed slightly reduced Akt phosphorylation on dSer505/mSer473 in *PP2A-B′* loss-of-function conditions in vivo and in cell culture (Figure 2D and Figure 4E, lanes 3 and 4). The only kinase known to phosphorylate S6K on Thr398 in *Drosophila* is TOR-C1. Therefore, increased phosphorylation of S6K in *PP2A-B′* knockout animals could be due to increased TOR-C1 activity. To test this, we assayed phosphorylation of another direct target of TOR-C1, 4E-BP. 4E-BP phosphorylation was not elevated in *PP2A-B′* knockouts (Figure 2B), indicating that elevated levels of S6K phosphorylation in KO1 animals are not due to increased TOR-C1 activity. Together, these results suggest PP2A-B′ acts specifically at the level of S6K.

The Human PP2A-B′ Homolog PPP2R5C Represses S6K1 Phosphorylation

Since the insulin pathway is highly conserved from flies to humans, we asked whether the human homologs of dPP2A-B′ also affect S6K phosphorylation. Alignment of human and *Drosophila* PP2A-B′ regulatory subunits reveals that dPP2A-B′ is most related to PPP2R5C and PPP2R5D (Figure S2A). Therefore, we tested both PPP2R5C and PPP2R5D. While siRNA-mediated knockdown of *PPP2R5D* had no consistent effect on S6K1 phosphorylation, knockdown of *PPP2R5C* with two independent siRNAs increased the amount of phosphorylated S6K protein in HeLa cells (Figures 2D and 2E and Figure S2B). Total levels of S6K1 protein were also elevated, perhaps as a secondary consequence of S6K1 stabilization (Wang et al., 2008) (Figures 2D and 2E). As in *Drosophila*, phosphorylation of 4E-BP and Akt were not increased (Figure 2D), indicating a specific effect on S6K1.

PP2A-B′ Interacts with S6K In Vitro and In Vivo

The specific effect of PP2A-B′ on S6K raised the possibility that PP2A-B′ might be acting directly on S6K. The regulatory subunits of PP2A such as PP2A-B′ are thought to provide specificity to the PP2A holoenzyme by recruiting it to specific substrates (Mumby, 2007). To test this, we asked whether PP2A-B′ and S6K interact physically. We expressed FLAG-tagged PP2A-B′ and HA-tagged S6K in S2 cells and precipitated

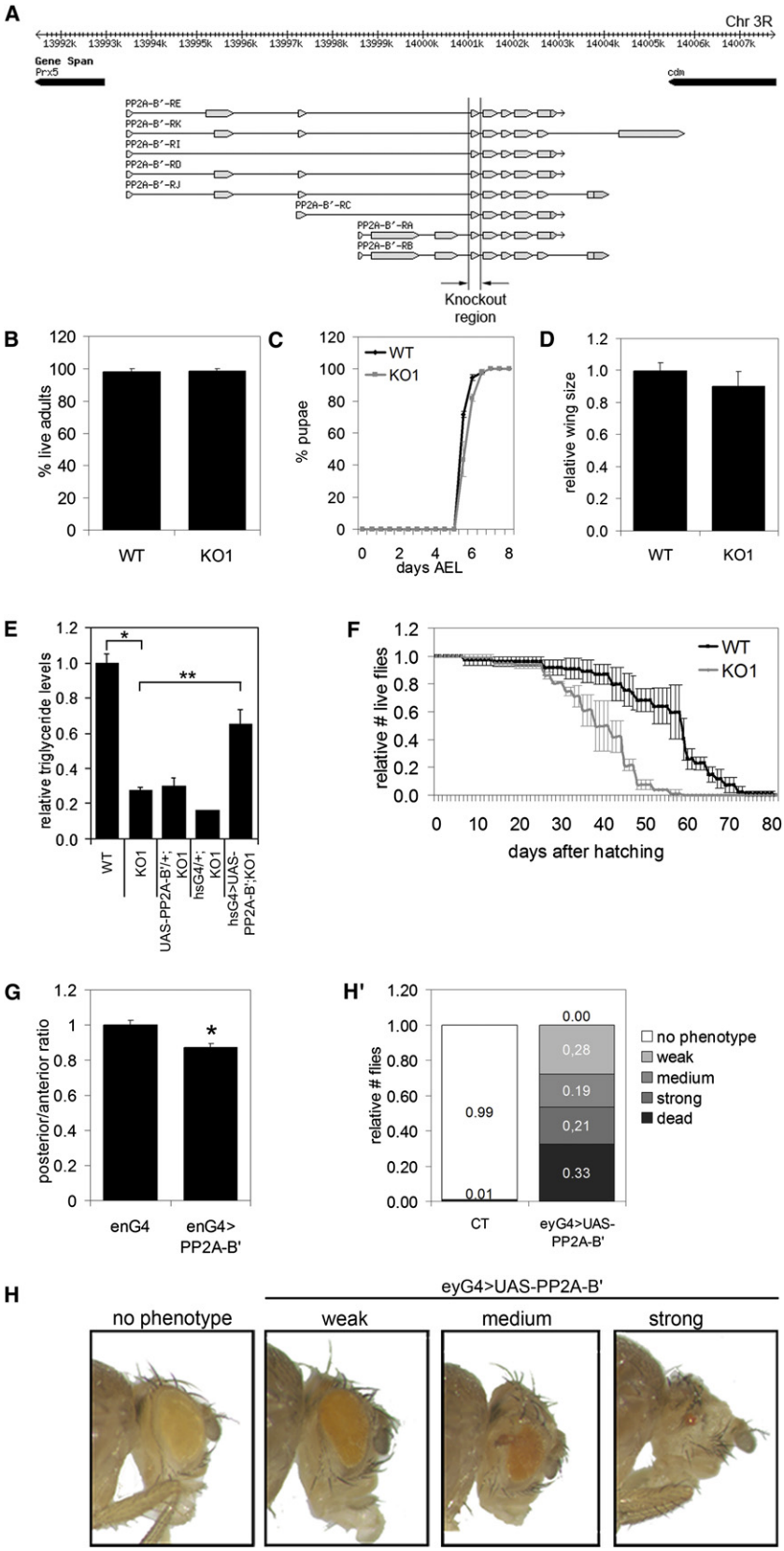

Figure 1. PP2A-B′ Knockout Flies Are Lean and Short Lived

(A) Overview of *PP2A-B′* genomic locus, showing the knockout region common to all splice forms.

(B–F) *PP2A-B′* loss-of-function phenotypes. w^{1118} control flies (WT) and KO1 knockout flies (KO1) were grown under controlled conditions on normal laboratory food at 25°C. (B) *PP2A-B′* knockout flies are viable. Shown is percentage of live adults relative to collected first-instar larvae. Error bars, standard deviation (SD). (C) *PP2A-B′* knockout flies are normal in developmental rate. Pupation curves show percentage of pupated animals over time. Error bars, SD (D) *PP2A-B′* knockout flies are almost normal in size. Shown is relative wing size of WT and KO1 flies. Error bars, SD (E) *PP2A-B′* knockout flies are lean. Shown are relative triglyceride levels, normalized to total body protein, for 5-day-old WT and KO1 flies, as well as KO1 flies carrying either a UAS-PP2A-B′ transgene, a heat shock GAL4 transgene (hs-G4), or both in order to express PP2A-B′ in the KO mutant background. Error bars, SD. *t test = 3×10^{-4}, **t test = 0.01. (F) *PP2A-B′* knockout flies have reduced life span. Mean life spans of WT and KO1 flies are 53.8 days (n = 97) and 38.5 days (n = 79), respectively. On day 50, 68% of WT and 8% of KO1 flies were alive (t test = 10^{-4}). Done in triplicate. Error bars, SD.

(G–H′) *PP2A-B′* overexpression reduces tissue size. (G) Posterior/anterior wing compartment size ratio of *engrailed*-GAL4 ("enG4") control or enG4 > PP2A-B′ overexpressing flies. Error bars, SD. *t test = 10^{-9}. (H) Phenotypes of flies overexpressing *PP2A-B′* using eyeless-Gal4, ranging from a mild to a strong reduction in size. (H′) Quantification of observed phenotypes from (H).

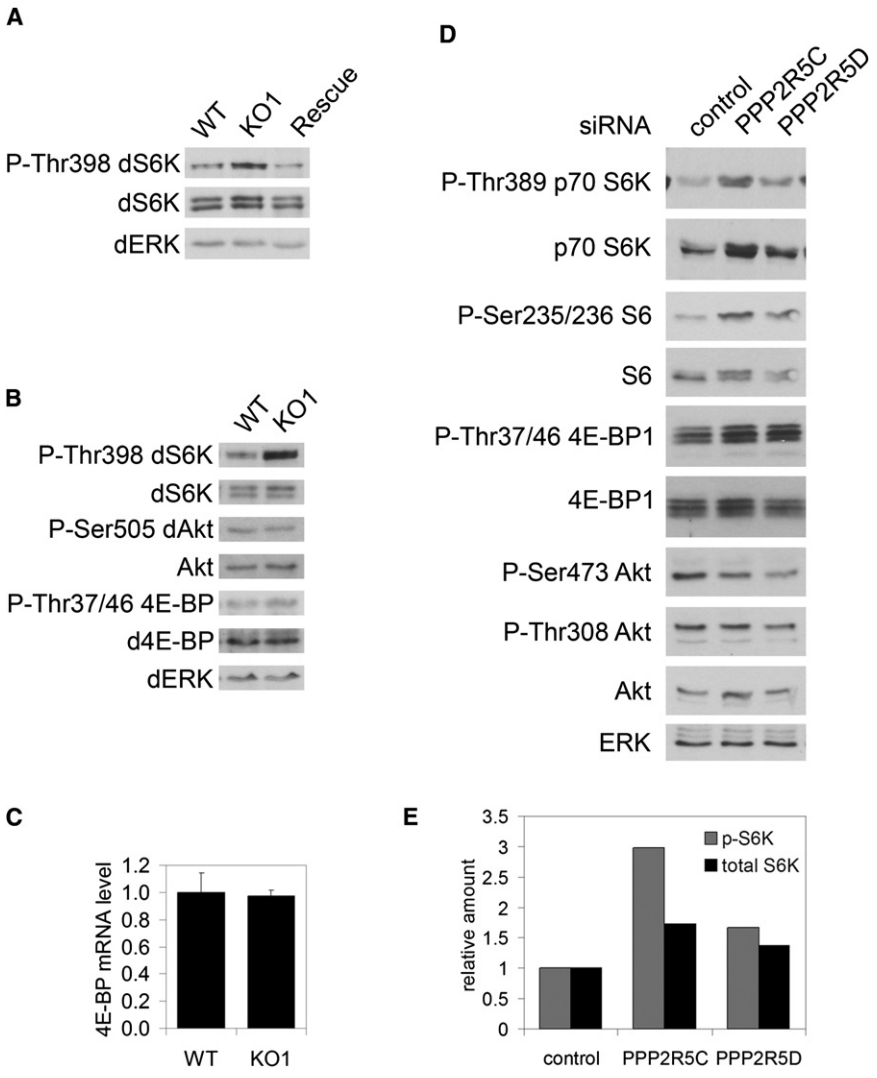

Figure 2. PP2A-B′ Represses S6K Phosphorylation in *Drosophila* and Humans

(A) *PP2A-B′* knockout animals have elevated levels of phosphorylated S6K protein. w^{1118} control (WT), KO1, and KO1 animals ubiquitously expressing *PP2A-B′* using heat shock Gal4, *UAS-PP2A-B′* transgenes ("rescue") were grown under controlled conditions. Wandering third-instar larval lysates were analyzed by immunoblotting for phospho-S6K and S6K protein as indicated. ERK was used as loading control.

(B) *PP2A-B′* knockouts have normal Akt and 4E-BP phosphorylation levels. Samples were prepared and processed as in (A).

(C) *PP2A-B′* knockout animals have normal FOXO activity. mRNA levels of the FOXO target 4E-BP in control (WT) and PP2A-B′ knockouts (KO1) were measured by quantitative RT-PCR relative to rp49. Error bars, SD.

(D) Knockdown of the mammalian PP2A-B′ homolog *PPP2R5C*, but not *PPP2R5D*, by siRNA increases S6K phosphorylation in HeLa cells. HeLa cells were transfected with Invitrogen negative control (control) or *PPP2R5C* or *PPP2R5D* siRNAs. ERK was used as loading control. *PPP2R5C* and *PPP2R5D* knockdown efficiency was 80% in both cases at mRNA level, measured by Q-RT-PCR.

(E) Densitometric quantification of phospho-S6K and total S6K bands shown in (D).

PP2A-B′ with anti-FLAG antibody. S6K could be detected in the FLAG immunoprecipitate (IP), suggesting PP2A-B′ and S6K interact physically (Figure 3A). S6K could not be detected in the FLAG IP in the absence of FLAG-PP2A-B′, demonstrating specificity of the pull-down (Figure 3A, lane 1). The S6K protein that coimmunoprecipitated with PP2A-B′ was found to be dephosphorylated (Figure S3A), consistent with the PP2A holoenzyme having acted upon it. In line with PP2A-B′ and S6K interacting physically, the two genes are coexpressed ubiquitously in fly tissues (Figures S3B and S3C).

To test whether the PP2A-B′-containing holoenzyme can dephosphorylate S6K directly in vitro, we expressed HIS-tagged S6K in *Drosophila* S2 cells, purified it on a nickel column, and eluted it with imidazole, yielding soluble, phosphorylated S6K that could be used as substrate for in vitro dephosphorylation assays. PP2A-B′ containing PP2A holoenzyme was purified by immunoprecipitation with anti-FLAG antibody from flies expressing FLAG-tagged PP2A-B′. As a control, we performed in parallel an anti-FLAG immunoprecipitation from flies not expressing FLAG-PP2A-B′. Whereas incubation of phospho-S6K with IP from control animals caused no dephosphorylation of S6K (Figure 3B, lanes 1 and 2), incubation with IP from FLAG-PP2A-B′-expressing animals caused strong and obvious dephosphorylation of S6K (Figure 3B, lane 3).

We tested whether PP2A-B′ and S6K interact genetically in vivo. If the phenotypes of *PP2A-B′* knockouts are due to elevated S6K activity, they should be rescued by removing one copy of *S6K* (KO1, $S6K^{+/-}$). Indeed, triglyceride and glycogen levels of KO1, $S6K^{+/-}$ flies were significantly rescued compared to KO1 flies (Figure 3C and Figure S1C). Removing one copy of *S6K* also partially rescued the decreased life span of *PP2A-B′* knockouts (Figure 3D). In summary, these results indicate that PP2A-B′ and S6K interact physically and genetically.

PP2A-B′ Knockouts Are Sensitive to Nutrient Conditions

While studying the *PP2A-B′* knockout flies, we realized that one of the *PP2A-B′* knockout lines, KO2, is semilethal when grown on standard laboratory food, which is rich in carbohydrates. Eighty-eight percent of KO2 animals die as pharate adults (Figure 4A). Both KO1 and KO2 lines were generated by backcrossing knockout flies for four generations to the w^{1118} stock, which is an inbred but not isogenic reference stock in the lab. The fact that KO2 is semilethal and KO1 is viable suggests the two lines differ in their genetic backgrounds. Indeed, the semilethality of KO2 flies could be completely rescued by changing the second chromosomes in the KO2 stock (Figure S4). This indicates the

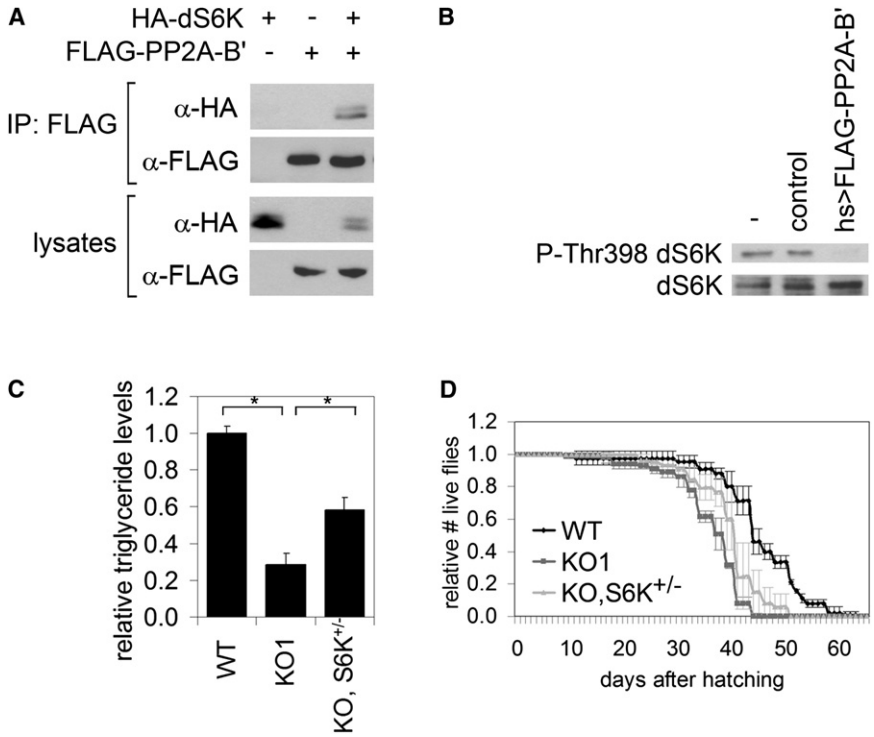

Figure 3. PP2A-B′ Interacts with S6K In Vitro and In Vivo

(A) PP2A-B′ interacts physically with S6K in S2 cells. Anti-FLAG immunoprecipitates (IP) and total lysates from S2 cells transfected to express HA-dS6K, FLAG-PP2A-B′, or both were probed with anti-FLAG and anti-HA antibodies. HA-dS6K can be detected in the anti-FLAG IP only in the presence of FLAG-PP2A-B′.

(B) PP2A-B′ dephosphorylates S6K in vitro. Phosphorylated HIS-tagged S6K purified from S2 cells was incubated with buffer (–), anti-FLAG IP from w^{1118} flies (control), or anti-FLAG IP from flies expressing FLAG-PP2A-B′ (hs > FLAG-PP2A-B). Phosphorylation and total levels of S6K were analyzed by Western blotting.

(C) Removal of one copy of *S6K* partially rescues the leanness of KO1 flies. Relative triglyceride levels, normalized to total body protein, of 5-day-old w^{1118} flies (WT), KO1 flies, and KO1 flies lacking one copy of *S6K* (KO, $S6K^{+/-}$). Animals were grown under controlled conditions. Error bars, SD. *t test $\leq$ 0.005.

(D) Removal of one copy of *S6K* partially rescues the shortened life span of KO1 flies. Mean life span of WT, KO1, and KO1, $S6K^{+/-}$ flies was 45.2 days, 35.9 days, and 39.6 days, respectively. On day 37, 51% of KO1 flies were still alive compared to 77% of KO1, $S6K^{+/-}$ flies (t test = 0.03). Done in triplicate. Error bars, SD.

semilethality is caused by genetic interaction between the *PP2A-B′* mutation on the third chromosome and genetic elements on the second chromosome. It is becoming increasingly clear that genetic background plays an important role in modulation of complex phenotypes and in development of diseases such as diabetes or cancer (Adamo and Tesson, 2008). This is well known in mice, for instance, where mutation of leptin (Lep^{ob}) in the C57BL/6J background causes transient hyperglycemia, subsiding 14–16 weeks of age, whereas the same mutation in the C57BLKS/J background causes severe diabetes with regression of islets and early death (Davis et al., 2005). Since one genetic background is not more "correct" than another, we decided to also study the KO2 flies. We first tested whether KO2 flies have the same metabolic and biochemical phenotypes as KO1 flies. KO2 animals, like KO1 animals, are lean (Figure 4B) and have elevated levels of S6K phosphorylation (Figure 4C). These results show that KO2 animals have the same metabolic and biochemical phenotypes as KO1 animals, except that they exhibit poor survival, indicating a more aggravated phenotype.

If the poor survival rate of KO2 flies is due to a combined effect of elevated S6K activation and other genetic background effects, reducing S6K phosphorylation by reducing insulin signaling should rescue them. We tested this by rearing animals on normal food (100% food) or on food diluted 1:5 with PBS/agarose to make it less rich (20% food). Animals growing on 20% food take several additional days to pupate compared to animals on 100% food, reflecting the lower richness of 20% food (data not shown), and accordingly have reduced expression of nutrient-responsive *insulin-like peptides 2* and *5* (Figure 4D) (Ikeya et al., 2002; Teleman et al., 2008). Consistent with this, animals growing on 20% food have reduced intracellular insulin signaling compared to animals on 100% food, assayed by phosphorylation of Akt and S6K (Figure 4E). On 20% food, levels of phospho-S6K in KO2 animals are still higher than in control flies (Figure 4E, lanes 3 and 4), consistent with phospho-S6K levels reflecting a balance of dephosphorylation by the PP2A/PP2A-B′ holoenzyme and nutrient-responsive phosphorylation by TOR. We then determined the survival rate of control and KO2 animals reared on 100% and 20% food. Intriguingly, on 20% food the survival of KO2 flies was significantly improved (Figure 4F). These results indicate that, unlike control flies, KO2 flies are sensitive to the nutritional overload of our rich laboratory food. This is particularly striking since 20% food is normally a stress condition for flies but results in improved viability here.

DISCUSSION

PP2A-B′ Counteracts S6K Phosphorylation

One reason phosphatases have been difficult to study is that they often act pleiotropically. Blocking their activity usually leads to multiple concurrent molecular effects and often lethality. The phosphatase PP2A is a case in point; it accounts for a large fraction of phosphatase activity in eukaryotic cells (Millward et al., 1999), and mutation of the PP2A catalytic subunit leads to lethality (Gotz et al., 1998). The PP2A active core dimer associates with a large number of possible B subunits that give it specificity by mediating interaction with specific targets (Mumby, 2007). Therefore, studies of the individual regulatory B subunits are critical for teasing apart the various functions of PP2A. We discover here the regulatory subunit of PP2A, PP2A-B′/PPP2R5C, as a novel component of the insulin pathway, which specifically directs PP2A to dephosphorylate S6K (Figure 2).

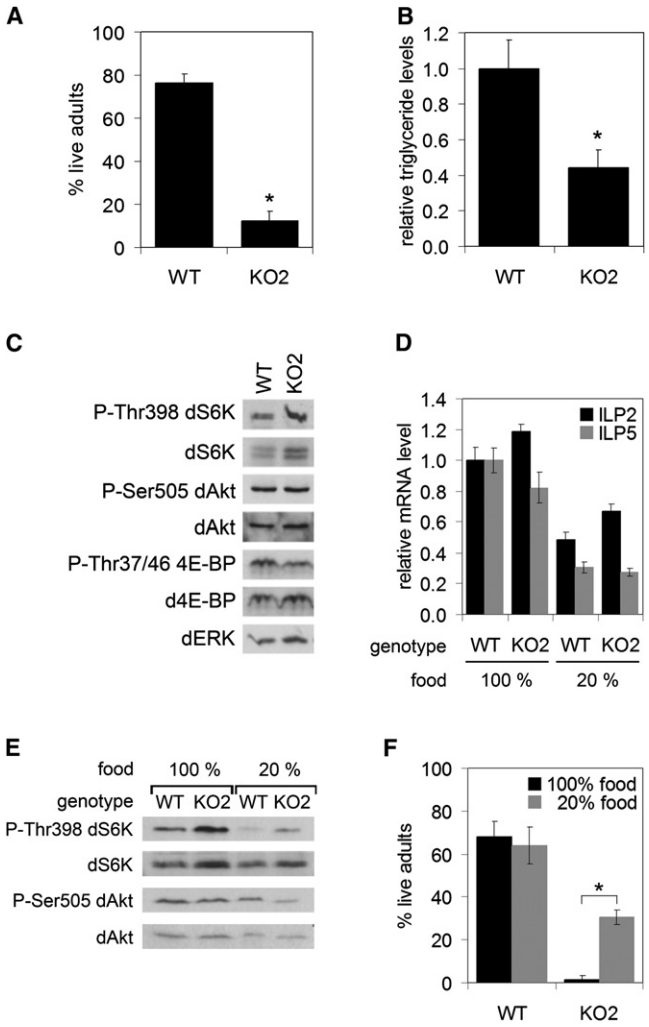

Figure 4. PP2A-B′ Knockout Flies Are Sensitive to Nutritional Conditions

(A) A second independent *PP2A-B′* knockout line (KO2) is semilethal. Percentage of live adult flies relative to collected first-instar larvae. Error bars, SD. *t test = 10^{-16}.

(B) Surviving KO2 adult flies are lean. Relative triglyceride levels, normalized to total body protein for animals grown under controlled conditions on normal laboratory food. Error bars, SD. *t test = 0.01.

(C) KO2 flies have elevated levels of S6K phosphorylation, but normal levels of 4E-BP and Akt phosphorylation, compared to WT. Wandering third-instar lysates were analyzed by immunoblotting as indicated. ERK was used as loading control.

(D–F) WT and KO2 animals were grown in parallel either on normal food (100%) or on food diluted 1:5 with PBS/agarose (20%). (D) Limiting food supply decreases insulin signaling in WT and KO2 animals. Quantitative RT-PCR of ILP2 and ILP5 mRNA from WT and KO2 wL3 normalized to rp49. Error bars, SD. (E) Nutrient deprivation lowers S6K phosphorylation in KO2 animals. wL3 lysates were analyzed by Western blotting as indicated. (F) Survival of KO2 flies is significantly increased when animals were reared on 20% food. Shown is percentage of live adult flies relative to collected first-instar larvae. Error bars, SD. *t test $\leq 10^{-4}$.

The phenotypes we observe in PP2A-B′ knockout flies—reduced longevity and reduced nutrient stores—are insulin gain-of-function phenotypes in the fly (Kapahi et al., 2004; Oldham et al., 2002) and are rescued by removal of one copy of S6K (Figures 3C and 3D and Figure S1C). Recently, the other B′ regulatory subunit, widerborst, and its corresponding homolog pptr-1 in *C. elegans* were shown to target PP2A to Akt (Padmanabhan et al., 2009; Vereshchagina et al., 2008), indicating that both B′ subunits are dedicated to keeping activation of the insulin signaling pathway under control.

S6K also regulates tissue growth (Hay and Sonenberg, 2004). Consistent with this, overexpression of *PP2A-B′* both in the wing and in the eye leads to a reduction in tissue size (Figures 1G and 1H), phenocopying *S6K* loss of function (Kozma and Thomas, 2002). Unexpectedly, *PP2A-B′* knockout flies are not larger than controls. One reason may be that S6K gain of function in the fly gives surprisingly mild overgrowth (Radimerski et al., 2002), suggesting S6K activity may not be limiting for fly growth under normal circumstances. Another reason may be that larval and imaginal tissues compete for nutrients during development and therefore the relative balance of PP2A-B′ activity in various tissues might be important. Future work may shed more light on this issue.

Flies mutant for *PP2A-B′*, called well-rounded, were recently reported with mild defects in the number and size of boutons at neuromuscular junctions (Viquez et al., 2006). TOR and S6K play a variety of roles in neuronal growth, differentiation, and dentritic growth and arborization. Whether repression of S6K phosphorylation by PP2A-B′ plays a role in this context remains to be investigated.

PP2A-B′ Is Required for Robustness to Nutritional Variation

Signaling through the insulin pathway is both activated and inactivated in a tightly controlled and rapid fashion, assuring adequate and timely responses to varying nutritional conditions. We show here that phosphatases, and in particular PP2A-B′, play a key role in maintaining this balance, as *PP2A-B′* mutants can become sensitive to nutritional status. The viability of KO2 flies varied dramatically depending on the richness of their food (Figure 4F), in contrast to wild-type animals, which were viable under a wide range of genetic backgrounds and nutritional conditions.

PPP2R5C and Human Disease

Drosophila PP2A-B′ has two mammalian homologs, PPP2R5C and PPP2R5D. Knockdown of *PPP2R5C* resulted in elevated levels of phosphorylated S6K protein in human cells (Figures 2D and 2E), suggesting PPP2R5C is the human functional homolog of PP2A-B′. Aberrant insulin and S6K signaling have been implicated in the etiology of a number of human diseases, including diabetes, obesity, and cancer (Hafen, 2004; Kozma and Thomas, 2002). Therefore, it will be interesting to investigate whether PPP2R5C, as a novel regulator of S6K phosphorylation, plays a role in development of these diseases. Our data suggest dPP2A-B′ is required for the organism to respond properly to elevated nutrient conditions. Diseases such as diabetes and obesity could be considered situations in which the ability of insulin signaling to compensate for elevated nutrient supply has been exceeded. Furthermore, S6K1, as one of the principal effectors of TOR-mediated tissue growth, has been linked to a number of cancers (Mamane et al., 2006). This raises the

possibility that PPP2R5C might also act as a tumor suppressor by inhibiting S6K1 phosphorylation. Indeed, PPP2R5C was identified in a screen for genes downregulated in malignant melanoma (Deichmann et al., 2001). Future work will shed further light on links between PPP2R5C and diseases involving insulin signaling, such as cancer and diabetes/obesity.

EXPERIMENTAL PROCEDURES

Expression Constructs, Fly Lines, and Metabolic Analyses

Detailed description of plasmid construction, oligos, and metabolic analysis is found in the Supplemental Information. HA-S6K expression construct was a kind gift from Duojia Pan. hs-Gal4, en-Gal4, eye-Gal4, and $S6K^{07084}$ flies were from Bloomington Stock Center.

Cell Culture, Transfection, IP, and Antibodies

A detailed description of cell-culturing conditions, IP, and antibodies is in the Supplemental Information. Gene knockdown in HeLa cells was performed in 6-well dishes using 5 μl of Dharmafect reagent (Dharmacon) and 0.1 μM of siRNAs (Dharmacon L-009433-00-005, L-009799-00-0005) or 0.07 μM of siRNA (Ambion P/N 4390824) per 1.5 × 10^5 cells/well, for 3 or 2.5 days, respectively. mRNA levels of target genes were reduced by 80%, quantified by Q-PCR.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at doi:10.1016/j.cmet.2010.03.015.

ACKNOWLEDGMENTS

We thank Stephen Cohen for comments on this manuscript. This work was supported by a Helmholtz Young Investigator grant. We apologize to the many authors whose work could not be cited due to space constraints.

Received: September 25, 2009
Revised: February 3, 2010
Accepted: March 24, 2010
Published: May 4, 2010

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